Cytokine-like protein 1-induced survival of monocytes suggests a combined strategy targeting MCL1 and MAPK in CMML

Margaux Sevin, Franck Debeurme, Séverine Badel, Margot Morabito, Hanna L Newman, Lucie Laplane, Miguel Torres-Martin, Qin Yang, Bouchra Badaoui, Orianne Wagner-Ballon, Véronique Saada, Dorothée Sélimoglu-Buet, Laurence Kraus-Berthier, Sébastien Banquet, Alix Derreal, Pierre Fenaux, Raphael Itzykson, Thorsten Braun, Gabriel Etienne, Celine Berthon, Sylvain Thepot, Oliver Kepp, Guido Kroemer, Eric Padron, Maria E. Figueroa, Nathalie Droin, Eric Solary

Supplemental Methods

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Supplemental Methods

Antibodies: Antibodies for western blotting include anti-MCL-1 (1:1000, clone D35A5), anti-Bcl-X_L (1:1000, clone 54H6), anti-PARP (1:1000), anti-phosho-ERK (1:2000, clone D13.14.4E), and anti-ERK (1:1000) from Cell Signalling (Danvers, Massachusetts); anti-Bcl2 (1:500, clone 100/D5) from Abcam (Cambridge, United kingdom), and anti-beta-actin-peroxidase (1:25000, clone AC15) and anti-HSC70 (1:200, clone B-6) from Sigma-Aldrich. Antibodies used for iBH3 profiling included Krome Orange anti-CD45 (1:100, clone J.33), ECD anti-CD24 (1:100, clone ALB9), PeCya7 anti-CD14 (1:100, clone RMO52), and APC anti-CD56 (1:100, clone N901) from Beckman Coulter and APC-H7 anti-CD16 (1:100, clone 3G8), PE anti-CD2 (1:100, clone RPA-2.10) from BD biosciences, and anti-cytochrome c (1:400) from Biolegend (San Diego, USA).

ChIP-qPCR: Cells were cross-linked with 1% formaldehyde for 10 min, fixation was stopped by addition of 125 mM glycin during 5 min before 2 washes in ice-cold PBS and addition of SDS lysis buffer (Millipore, 10 uL per 1×10^6 cells) supplemented with 1% protease inhibitor cocktail (Active Motif). Samples were incubated for 15 min on ice before 10 min sonication at 40 W (Covaris S220, Woodingdean, UK). ChIP was carried out using ChIP-it express kit (Active Motif). Quantitative real-time PCR was performed using SYBR-Green (ThermoFisher Scientific) in an applied biosystem 7500 thermocycler (Applied Biosystems, ThermoFisher Scientific, 2 µL of ChIP or IgG or input DNA, 50 nM (each) primers, and 1× SYBR-Green mixture in 20 µl). Two sets of primers inside h*CYTL1* enhancer were used:

CYTL1 enhancer, set 1:

- forward: 5'-CGGCCTCATGGAAGGAAGA-3';
- reverse: 5'-GCAATCAAGGGCATCATTCA-3';

CYTL1 enhancer, set 2:

- forward: 5'-GCAGGAAGTGGGTGAGAACAAC-3';
- reverse: 5'-GCTGAGCCAGTTTCCGAATT-3'.

***EGR1* knockdown:** We used Stealth siRNAduplex (ThermoFisher Scientific) targeting *EGR1* and Stealth RNAi™ siRNA negative control introduced into U937 by nucleofection (Lonza, 5×10^6 cells in 100 µl nucleofector solution with 1nmol siRNA). siRNAs targeting EGR1:

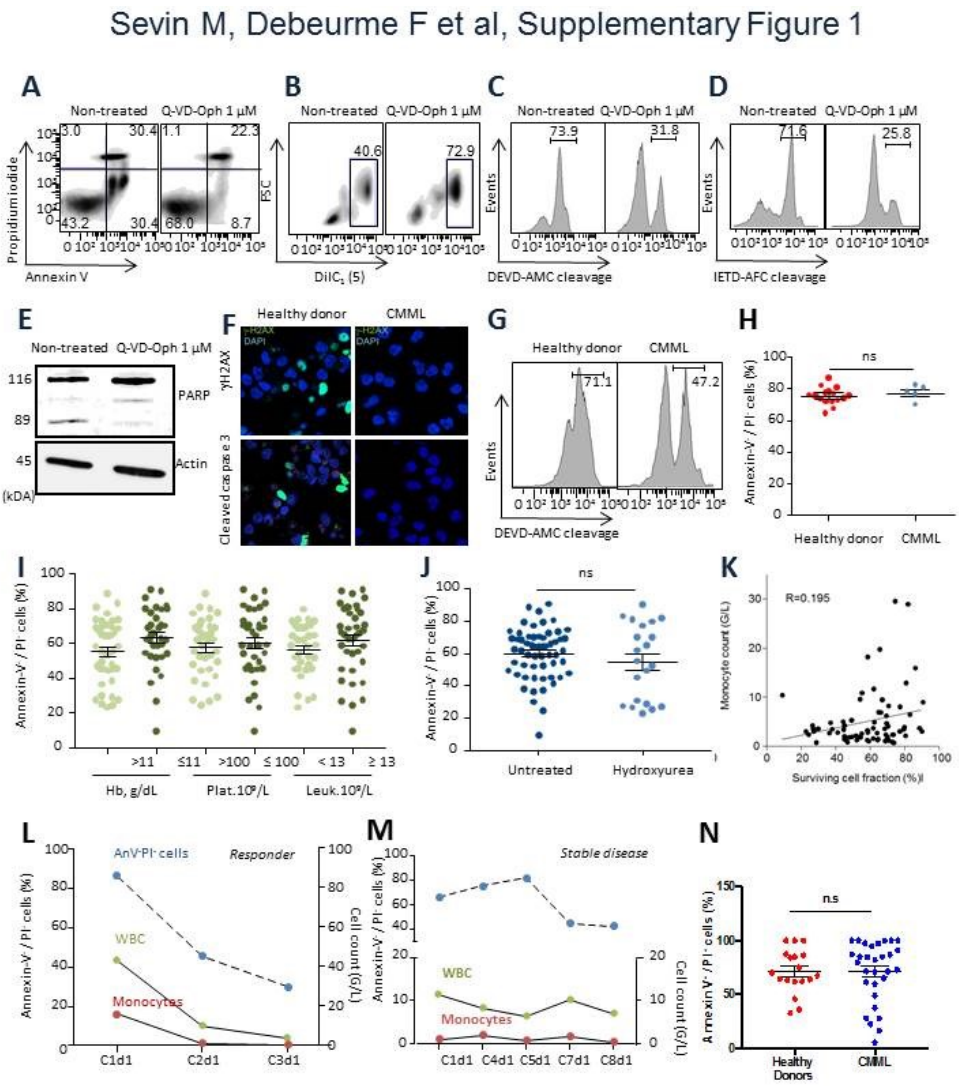
- #1: 5'-UCUCCCAGGACAAUUGAAAUUUGCU-3';
- #2: 5'-AGCAAAUUUCAAUUGUCCUGGGAGA-3';
- #3: 5'-GAUCUCUGACCCGUUCGGAUCCUUU-3'.

Knock-down efficacy checked using RT-qPCR

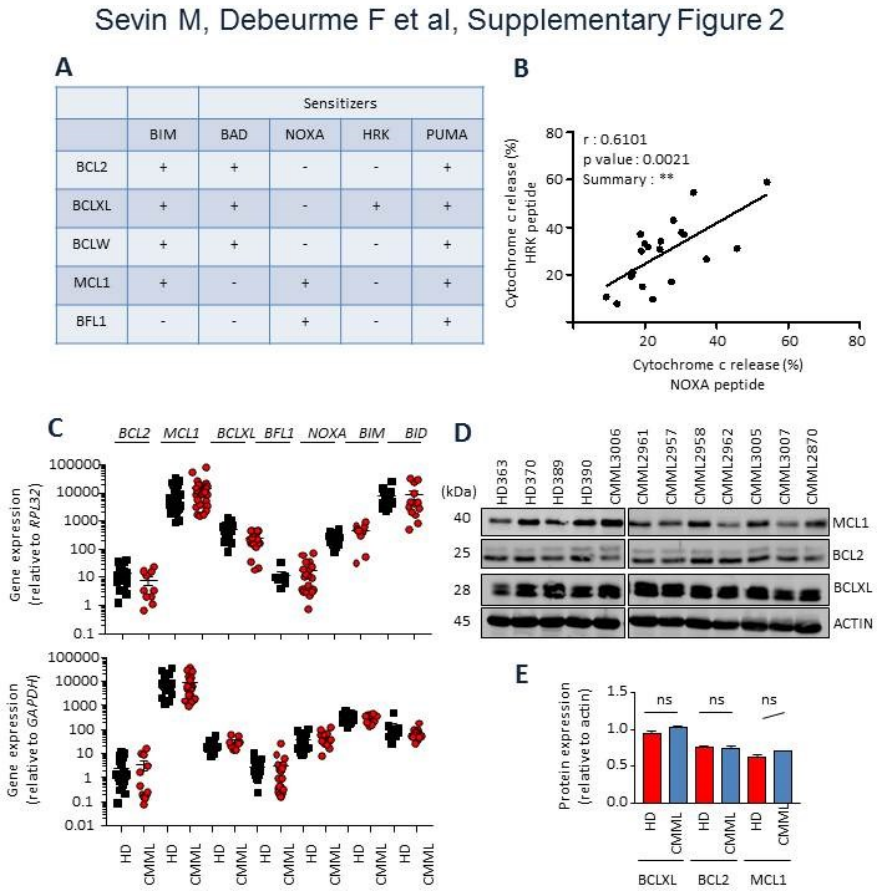
- forward: 5'-TGATGTCCCCGCTGCAG-3'
- reverse: 5'-GTCCATGGTGGGCGAGTG-3'

Patient-derived xenografts (PDX). Experiments were performed in NOD-scid IL2Rgnull-3/GM/SF (NSGS) mice. In a first series of experiments, NSGS mice (Charles River France, L'Arbresle, France) were housed in accordance with institutional standards set by Gustave Roussy Cancer Center. Animals aged 8-15 weeks were sub-lethally irradiated before injection of patient bone marrow mononucleated cells (3.5×10^6) or sorted $CD34^+$ cells (0.4×10^6) in 200 µL PBS via retro-orbital venous sinus. Seven weeks later, these animals were treated for 5 days per week with 1.0 mg/kg trametinib or 10 mg/kg selumetinib (oral gavage) and once per week with 20 mg/kg S63845 intravenously. After 3 weeks, animals were sacrificed, tissue supernatants and plasma samples were collected, cells were stained with APC anti-hCD45 and FITC anti-mCD45 (BD Biosciences) and acquired on LSR FortessaTM.

In the second series of experiments, NSGS mice bred under pathogen-free conditions (JAX Stock #013062) were housed in accordance with institutional standards set by Moffitt Cancer Center and the University of South Florida. All supporting procedures were performed with approval by the institutional animal care and use committee of the University of South Florida (IACUC protocol IS00006041). Two to four million, T-cell depleted, bone marrow mononuclear cells (BMNCs) from 4 unique CMML patient samples were transplanted via tail vein into 12 sub-lethally irradiated, 6 to 10-week-old female NSGS mice. Ten-14 days post-transplant, mice were randomized into four equal groups to receive: vehicle, trametinib, S63845, or combination. Mice were treated for two weeks and then sacrificed. Trametinib was administered orally at 1mg/kg once a day for five days a week, while S63845 or vehicle was administered at 20mg/kg via a single intravenous injection once per week for a total of 3 injections. Splenocytes, bone marrow, and peripheral blood were isolated post-mortem. Leukemic engraftment was calculated by flow cytometry using an LSR II (BD Sciences, Franklin Lakes, New Jersey). All gating was done to exclude non-singlets and dead cells. Engraftment was defined by the percentage of human $CD45^+$ cells (BD Cat No: 564047, clone: HI30). FACS results were analyzed by FlowJo v8 (RRID:SCR008520) software.

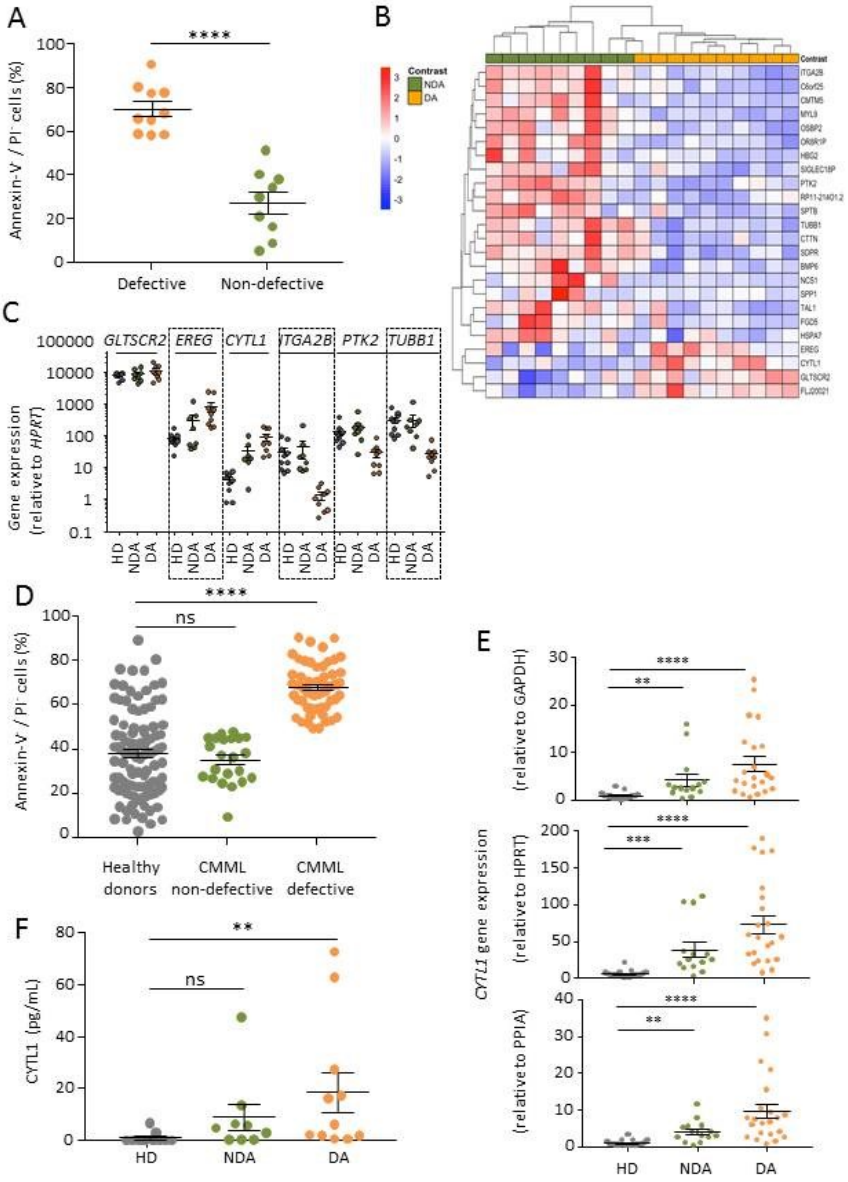


Supplemental figure 1. Monocyte apoptosis. A to F. Healthy donor monocytes were cultured in RPMI 1640 medium with 10% FCS for 24 hours in the absence of presence of 1 µM Q-VD-Oph before measuring the fraction of annexin-V-negative, propidium iodide–negative cells (**A**), the fraction of DiIC1 high cells (**B**), the cleavage of the caspase substrates DEVD-AMC (**C**) and IETD-AFC (**D**), the cleavage of poly(ADP-ribose)polymerase protein by immunoblotting (**E**), the cells showing a γH2AX nuclear ring and active caspase-3 (**F**). **G.** Comparison of DEVD-AMC cleavage by healthy donor and CMML sorted monocytes after 24 hours in culture as above. **H.** Fraction of annexin-V negative, propidium iodide–negative (AnV^-PI^-) cells in peripheral blood of healthy donors and CMML patients, immediately after sample collection. Mann-Whitney test, ns=non-significant. **I**. AnV^+/PI^- fraction was measured as in C in $CD14^+$ monocytes sorted from untreated (n=58) or hydroxyurea (HU) treated (n=21) CMML patient. **J**. Fraction of surviving cells shown in panel I according to indicated biological parameters. **K.** Correlation between cell surviving fraction (AnV^-/PI^- fraction of CMML $CD14^+$ monocytes after 24 hours in culture with 10% FCS, n=78) and the blood monocyte count. **L,M.** Evolution of the fraction of AnV^-PI^- monocytes after 24 hours in culture, white blood cell count (WBC) and monocyte count in the peripheral blood after indicating cycles of azacytidine treatment in a responding CMML patient (**L**) and a patient whose disease remained stable upon therapy (**M**). **N.** AnV^-PI^- fraction of $CD34^+$ cells after 24 hours in serum containing culture medium without additional cytokines (healthy donors, n=18; CMML, n= 30).



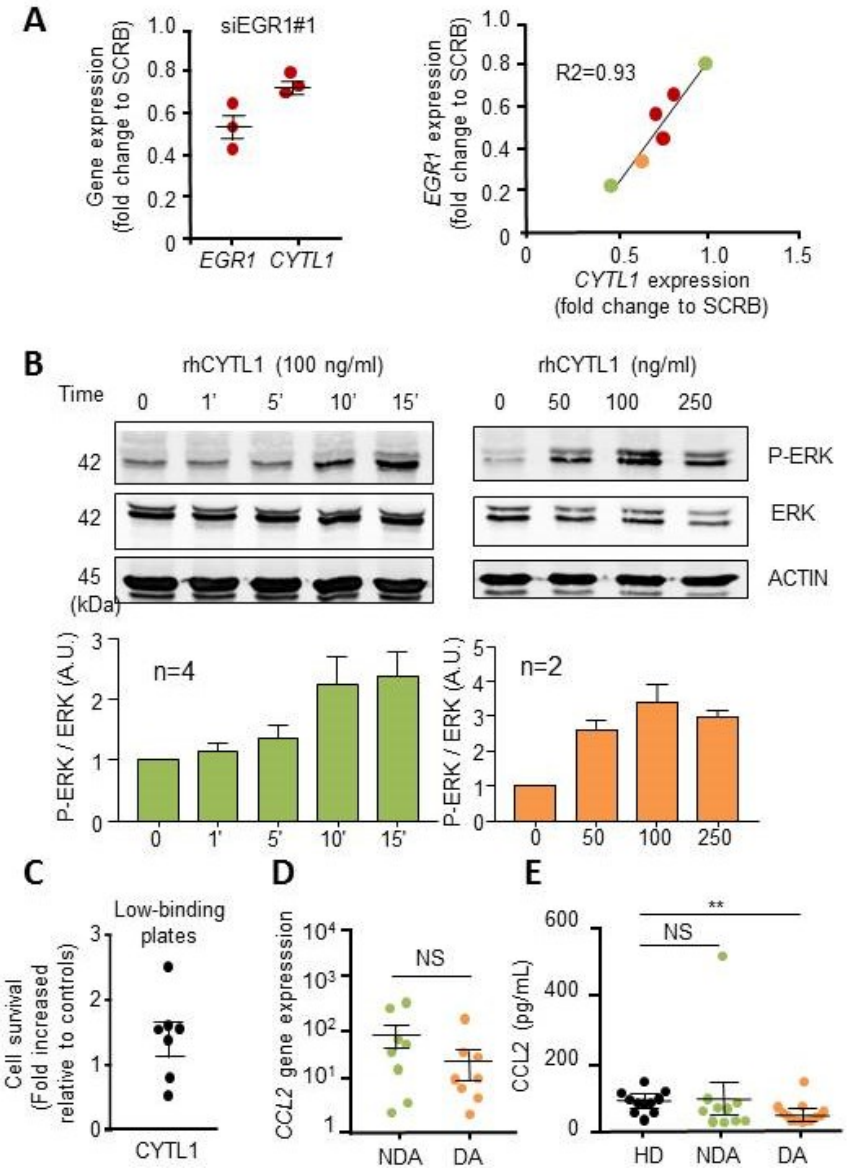
Supplemental figure 2. CMML monocyte addiction to MCL1. A. Selective binding of BH3 peptides to proteins of the BCL2 family, based on fluorescent polarization binding studies used in the iBH3 profiling assay; +, binding, – no binding. **B.** Relationship between NOXA and HRK-induced apoptosis of CMML patient monocytes. **C.** RT-qPCR quantification of indicated genes of the BCL2 family in sorted healthy donor and CMML patient monocytes using two different endogenous control genes, *GAPDH* and *RPL32*. **D.** Immunoblot analysis of indicated proteins in sorted monocytes collected from 4 healthy donors and 8 CMML patients. **E.** Immunoblot quantification using imageQuant LAS 4000 camera and ImageJ software (n=3; mean ± SEM).

Sevin M, Debeurme F et al, Supplementary Figure 3



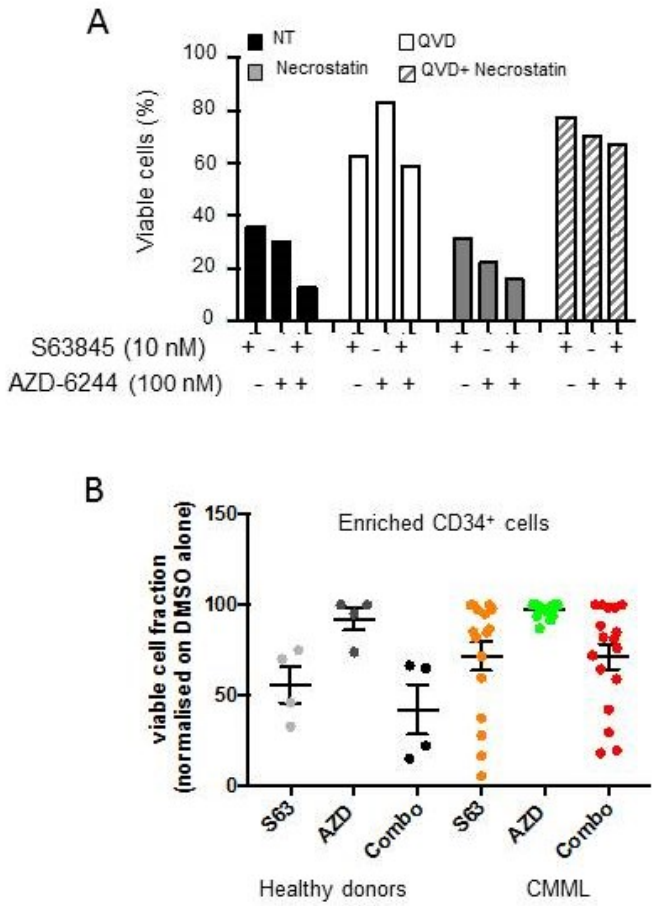
Supplemental figure 3. CYTL1 is overproduced by CMML monocytes. A. CMML patients samples were arbitrarily separated into two groups, based on their sensitivity to apoptosis when cultured for 24 hours in serum containing medium (defective, less than 50% of dead cells, non-defective, less than 50% of live cells). Mann-Whitney test, **** *P*<0.0001. **B.** Heatmap of differentially expressed gene between samples with defective and non-defective apoptosis. **C.** RT-qPCR validation of indicated genes on samples used to perform RNA sequencing analysis. **D.** Cohort of healthy donor and CMML patient monocytes tested for their sensitivity to apoptosis and classified as in A, suggesting that non-defective CMML samples are close to controls while defective CMML samples are significantly distinct. **E.** Validation of CYTL1 gene overexpression in cohorts of CMML samples with defective and non-defective apoptosis compared to healthy donor sorted monocytes. **F.** CYTL1 plasma concentration in healthy donors and patients with CMML whose monocytes are classified as defective or non-defective apoptosis as in A. **D,E,F,** Kruskall-Wallis test, Dunn's multiple comparison test, ** *P*<0.01, *** *P*<0.001, **** *P*<0.0001; HD, healthy donors; DA, defective apoptosis; NDA, non-defective apoptosis.

Sevin M, Debeurme F, Supplementary Figure 4



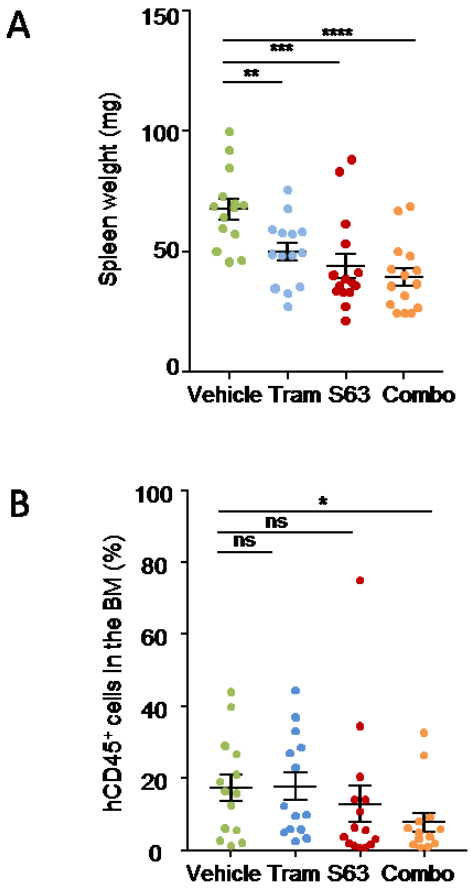
Supplemental figure 4. EGR1-regulated CYTL1 activates ERK in human monocytes. A. siRNA-mediated down-regulation of EGR1 in U937 cells: left panel, a first siRNA (red), tested in triplicate, induced a decrease in both *EGR1* and *CYTL1* gene expression; right panel, correlation between *EGR1* and *CYTL1* gene expression decrease induced by three distinct siRNAs (colors) targeting *EGR1* (Spearman test). **B.** Healthy donor monocytes were treated with 100 ng/ml rhCYTL1 for indicated times or with indicated concentration of CYTL1 for 15 minutes before exploring the phosphorylation of ERK by immunoblotting of indicated proteins. Lower panel show the mean ± SEM of indicated number of independent experiments. **C**. Healthy donor monocytes were cultured on low-binding plates for 24 hours in serum-free medium in the absence or presence of 100 ng/ml rhCYTL1 before measuring the fraction of AnV^-/PI^- cells (survival increase in CYTL1-treated relative to untreated cells). D. CCL2 gene expression in monocytes with non-defective (NDA) and defective (DA) apoptosis analyzed by RNA sequencing on Figure 3C; **E.** CCL2 cytokine level in the supernatant of monocytes sorted from health donors (HD) and CMML patiensts (NDA and DA as on panel D).

Sevin M, Debeurme F et al, Supplementary Figure 5



Supplemental figure 5. The S53845/MEK inhibitor combination induces monocyte death by apoptosis. A. $CD14^+$ monocytes sorted from CMML patient peripheral blood were treated with S63845 (10 nM) and/or AZD6244 (100 nM) in the presence or absence of a necroptosis (necrostatin, 50 µM) or an apoptosis (Q-VD-OPH, 50 µM) inhibitor for 24 hours. The surviving fraction of cells (AnV^{neg};IP^{neg}) was determined by flow cytometry. **B.** Enriched $CD34^+$ cells collected from CMML patients or healthy donors were treated similarly with S63645, AZD6244 and the combination (combo) for 24 hours before measuring cell viability normalized to DMSO treatment.

Sevin M, Debeurme F et al, Supplementary Figure 6



Supplemental figure 6. Figure 6. The S63845 / Trametinib combination decreases leukemic infiltration in CMML PDX models. We pooled results shown on Figure 6E (8 animal per groups) with those obtained in two additional PDX models (6 animals per groups)**.** The impact of the trametinib, S63845 and the combination on spleen weight (A) and the fraction of hCD45+ cells in the bone marrow (B) is shown * *P*<0.05; ** *P*<0.01; *** *P*<0.001; **** *P*<0.0001 (Mann-Whitney test).

Supplemental table 1. Primers used for RT-qPCR experiments.

Gene	For/Rev		Tm (°C)	GC (%)	Product length (bp)	Target on primer blast
CYTL1	F	CAGGCTGTACCTGGACATACACA	59	52	67	NM_018659
CYTL1	R	GGGCGAGGCCACAAAGT	58	65	67	
Bcl2	F	AACTGTACGGCCCCAGCAT	61.6	57.9	73	NM_000633.3
Bcl2	R	GCCAAACTGAGCAGAGTCTTCAG	61.7	52.2		
BCLXL/XS	F	GAA CGG CGG CTG GGATA	59.1	64.7	58	NM_001317919.2
BCLXL/XS	R	GCTCTCGGCTGCTGCATT	60.82	61.1		
BFL1	F	CAGGAGAATGGATAAGGCAAA	59	43	64	NM_001114735.2
BFL1	R	ACCAGCATAGGTGTGTGATTGT	59	45		
NOXA	F	GGAGATGCCTGGGAAGAAG	59	58	94	NM_021127.3
NOXA	R	CCTGAGTTGAGTAGCACACTCG	60	55		
BIM	F	GCAGATATGCGCCCAGAGAT	60.04	55	82	NM_001204106.2
BIM	R	CCCTCCTTGCATAGTAAGCGT	59.86	52.38		
BID	F	GCC TTT ATT TCC ACT GTG CAG G	60.09	50	147	NM_001196.4
BID	R	TGC CTG CTG TAA GAC CAT CC	59.75	55		
GAPDH	F	AAGGTCGGAGTCAACGGGT	60.8	57.9	63	NM_001357943.2
GAPDH	R	ACACTTAAAAGCAGCCCTGGTG	61.3	50		
Mcl-1	F	CGTTGTCTCGAGTGATGATCCA	60.16	50	63	NM_021960.5
Mcl-1	R	TCACAATCCTGCCCCAGTTT	59.52	50		
PPIA	F	GTCGACGGCGAGCCC	59.85	80	218	NM_021130.5
PPIA	R	TCTTTGGGACCTTGTCTGCAA	59.79	47.62		
HPRT	F	TTTGAAATTCCAGACAAGTTTGTTG	60.84	57.89	63	NM_000194.3
HPRT	R	CCAGTTTCACTAATGACACAAACATGA	61.34	50		
L32	F	TGTCCTGAATGTGGTCACCTGA	61.3	50	79	NM_031903.3
L32	R	CTGCAGTCTCCTTGCACACCT	62.6	57		

Supplemental table 2. Characteristics of patients included in each experiment. Cohorts used correspond to indicated panels. M, male; F, female; CMML, chronic myelomonocytic leukemia; Karyotype: N, normal; A, abnormal; NA, not available.

	Figure 1			Figure 2				Figure 3 et supplemental figure 3				Figure 5
	Cohort #1 Panel 1A	**Cohort #2 Panel 1B**	**Cohort #3 Panel 1 C-G**	**Cohort #4 Panel 2A**	**Cohort #5 Panel 2B**	**Cohort #6 Panel 2 C & D**	**Cohort #7 Panel 2E**	**Cohort #8 Panel 3A/C S3A/B**	**Cohort #9 Panel 3D**	**Cohort #10 Panel 3E S3E**	**Cohort #11 Panel 3G**	**Cohort #12 Panel 5A/B**
Number of patients	16	21	78	21	30	8	89	19	19	35	20	60
Mean age, yrs (range)	72.9 (28-88)	73.86 (58-90)	74.05 (51-90)	76.8 (29-90)	77.4 (65-90)	77 (66-88)	74.1 (48-90)	70.2 (53-86)	71.1 (50-91)	72.8 (53-86)	75 (61-85)	72.8 (39-100)
Sex ratio M/F	10/6	11/9	48/28	11/10	16/13	7/1	50/36	12/5	9/9	22/12	16/4	33/25
CMML 0 / 1 / 2	10/1/5	14/1/6	39/19/16	17/3/1	17/10/2	2/3/3	32/33/12	3/6/7	6/3/4	16/08/2007	5/9/4	11/29/13
Proliferative / dysplastic	8/8	10/9	39/36	11/10	15/15	2/5	38/40	13/3	7/10	22/10	9/7	25/24
Leucocytes, mean 10^9/L (range)	14.5 (3.8-43.8)	20.6 (4.1-44.5)	19.8 (4-119)	13.04 (4.6-32.02)	17.2 (4.6-77.1)	16.5 (6-57.5)	22.3 (4.3-137.3)	29 (5.8-119)	22.7 (4.3-102.6)	24.8 (4-119)	17.3 (4.28-42.9)	20.5 (4.6-86.1)
Monocytes, mean 10^9/L (range)	3.4 (1-9.1)	6 (0.6-8)	5.1 (0.7-29.6)	2.3 (0.4-5.7)	3.7 (1.2-9.6)	2.9 (1.1-6.3)	4.5 (0-29)	6.5 (1.4-19.7)	4.2 (1-15.9)	6.5 (08-29.6)	4.16 (1.6-15.9)	5.4 (0.7-39.6)
Plat. count, mean 10^9/L (range)	130.8 (34-285)	151,3 (14-451)	163.5 (25-996)	169.6 (35-674)	130 (23-597)	181.7 (98-246)	152.2 (10-622)	117.9 (18-417)	131 (25-440)	157.4 18-451)	191.2 (25-996)	130.1 (18-465)
Hemoglobin, mean g/dL (range)	12 (7.1-15.4)	11.3 (7.3-15.6)	11.5 (6.8-16.8)	12.6 (8.3-16.7)	12.1 (7.7-15.9)	11.8 (9.5-14.8)	11.4 (6.8-17)	11.3 (7.6-15.3)	10.8 (7.5-15.3)	11.6 (7.3-15.3)	11.5 (7.5-16.8)	11.6 (8.2-17)
Karyotype (N/A/ND)	13/3/0	13/1/7	50/17/11	17/4/0	23/6/1	2/2/4	51/12/26	13/1/5	12/3/4	20/6/9	16/4/0	29/9/22
Gene mutations; detected / tested (%)												
TET2	12/16 (73%)	16/21 (76%)	56/77 (73%)	15/18 (83%)	23/29 (79%)	4/6 (67%)	61/82 (74%)	13/19 (68%)	13/19 (68%)	22/34 (64%)	13/19 (68%)	36/51 (71%)
SRSF2	3/13 (23%)	10/21 (48%)	35/77 (45%)	5/17 (29%)	12/29 (43%)	2/6 (33%)	32/79 (41%)	7/18 (39%)	5/19 (26%)	14/33 (42%)	6/18 (33%)	19/48 (40%)
ASXL1	3/15 (20%)	7/21 (33%)	34/76 (46%)	4/19 (21%)	8/28 (29%)	1/6 (17%)	30/83 (36%)	7/19 (37%)	8/18 (44%)	15/35 (43%)	11/19 (58%)	21/51 (41%)
NRAS	2/15 (13%)	7/21 (33%)	16/77 (21%)	1/19 (5%)	5/29 (17%)	1/6 (17%)	16/82 (19%)	3/19 (16%)	3/19 (16%)	9/35 (26%)	5/19 (26%)	9/51 (18%)
KRAS	0/15 (0%)	1/21 (5%)	12/77 (16%)	2/19 (10%)	4/28 (14%)	0/6 (0%)	10/82 (12%)	5/19 (26%)	2/19 (11%)	8/35 (23%)	1/19 (5%)	7/51 (14%)
CBL	1/15 (7%)	3/21 (14%)	10/77 (13%)	3/19 (16%)	8/29 (28%)	1/6 (17%)	10/80 (13%)	0/19 (0%)	4/19 (21%)	1/35 (3%)	4/19 (21%)	5/50 (10%)

Supplemental table 3. Sequence of peptides used for iBH3 profiling experiments.

Peptide Name	Sequence
hBIM	Ac-MRPEIWIAQELRRIGDEFNA-NH2
hBID-Y	Ac-EDIIRNIARHLAQVGDSMDRY-NH2
mBAD	Ac-LWAAQRYGRELRRMSDEFEGSFKGL-NH2
mNoxaA	Ac-AELPPEFAAQLRKIGDKVYC-NH2
Puma	Ac-EQWAREIGAQLRRMADDLNA-NH2
Bmf-Y	Ac-HQAEVQIARKLQLIADQFHRY-NH2
W-Hrk	Ac-WSSAAQLTAARLKALGDELHQ-NH2
Puma2A	Ac-EQWAREIGAQARRMAADLNA-NH2

Supplemental table 4. Main differentially expressed genes between CMML monocytes with defective and non-defective apoptosis, as identified by RNA sequencing.

Ensg	Gene_symbol	baseMean	log2FoldChange	fold	lfcSE	stat	pvalue	padj
ENSG00000005961.13	*ITGA2B/CD41*	193,4956591	-2,330568893	0,198805711	0,30152366	-7,72930686	1,08E-14	2,24E-10
ENSG00000101335.5	*MYL9*	13,5746427	-2,030104691	0,244837307	0,341984567	-5,936246497	2,92E-09	3,02E-05
ENSG00000169398.15	*PTK2*	394,1443303	-1,904301313	0,267145697	0,328261084	-5,801179009	6,59E-09	4,55E-05
ENSG00000254531.1	*AP001816.1*	95,08138122	1,084412594	2,120511922	0,190973379	5,678344268	1,36E-08	7,05E-05
ENSG00000118785.9	*SPP1*	40,98283879	-1,92580161	0,263193979	0,349174961	-5,515291264	3,48E-08	0,000144455
ENSG00000166091.15	*CMTM5*	15,50873587	-1,804444337	0,286291286	0,350017168	-5,155302375	2,53E-07	0,000875431
ENSG00000101162.3	*TUBB1*	197,584521	-1,704094557	0,306913805	0,33418719	-5,09922165	3,41E-07	0,001010637
ENSG00000184792.11	*OSBP2*	35,75400885	-1,650793367	0,318464978	0,344090477	-4,797556101	1,61E-06	0,0041645
ENSG00000153162.8	*BMP6*	21,28943529	-1,651157599	0,318384587	0,346445699	-4,765992482	1,88E-06	0,004331283
ENSG00000070182.13	*SPTB*	23,72638817	-1,661177663	0,316180947	0,350918894	-4,733793744	2,20E-06	0,004570962
ENSG00000204420.4	*C6orf25*	44,96626098	-1,600127398	0,329847849	0,344326719	-4,647119462	3,37E-06	0,0063474
ENSG00000256347.1	*OR8R1P*	13,31474823	-1,408479061	0,376708617	0,32470833	-4,337674548	1,44E-05	0,024891283
ENSG00000085733.11	*CTTN*	33,49912275	-1,505935485	0,352101802	0,349358838	-4,310569305	1,63E-05	0,025982169
ENSG00000162367.7	*TAL1*	63,51953037	-1,487238473	0,356694661	0,348785661	-4,26404706	2,01E-05	0,029745037
ENSG00000124882.3	*EREG*	1868,228444	1,234583827	2,35313457	0,290639822	4,247813736	2,16E-05	0,029851493
ENSG00000168497.4	*SDPR*	130,808603	-1,375312062	0,385469318	0,326628117	-4,210635854	2,55E-05	0,033014161
ENSG00000107130.6	*NCS1*	4,630721749	-1,414864442	0,375044986	0,337337208	-4,194214001	2,74E-05	0,033410797
ENSG00000266709.1	*RP11-214O1.2*	23,71717768	-1,433702546	0,37017964	0,344288539	-4,164247089	3,12E-05	0,035998517
ENSG00000154783.6	*FGD5*	14,39523364	-1,452212917	0,365460422	0,349868041	-4,150744695	3,31E-05	0,036079327
ENSG00000170891.6	*CYTL1*	50,99551242	1,359973529	2,566804698	0,328525564	4,139627707	3,48E-05	0,036079327
ENSG00000105373.14	*GLTSCR2*	8711,221865	0,575819459	1,490523849	0,141378399	4,072895604	4,64E-05	0,044672683
ENSG00000196565.8	*HBG2*	8,502898038	-1,41625066	0,374684795	0,349876003	-4,047864528	5,17E-05	0,044672683
ENSG00000225217.1	*HSPA7*	507,430734	-1,277789438	0,412426964	0,315612535	-4,048601674	5,15E-05	0,044672683
ENSG00000268581.1	*SIGLEC18P*	21,95472147	-1,364455784	0,388380916	0,336081787	-4,059892077	4,91E-05	0,044672683